Remarks

Claims 25-46 are pending in the instant application.

Applicants have canceled claims 1, 13, 15, 17-20, 22 and 24 without prejudice or disclaimer. Applicants reserve the right to pursue the canceled subject matter in one or more continuing applications. Applicants have also herein amended claims 37 and 42 to recite the phrase "consisting of." No new matter has been added.

I. Claim Rejections Under 35 U.S.C. §§ 101/112

The Examiner has rejected claims 15 and 25-46 under 35 U.S.C. § 101 because the claimed invention is allegedly not supported by either a specific and substantial asserted utility or a well-established utility. In particular, the Examiner alleges, "the application is devoid of description of utility and working examples of the presently claimed protein function which is neither clearly defined nor demonstrated." *See* Paper No. 9, page 4, last line to page 5, line 2.

Applicants respectfully disagree and traverse.

Preliminarily, Applicants point out that claim 15 has been canceled, thus rendering the rejection to claim 15 moot. Applicants respectfully request withdrawal of the rejection to claim 15.

In order to find that an asserted utility is neither specific nor substantial, the burden is on the Examiner to make a *prima facie* case showing that it is more likely than not that a person of ordinary skill in the art would not consider any utility asserted by the Applicant to be specific or substantial. *See* M.P.E.P. § 2107.02(IV); Utility Examination Guidelines, 66 FR 1092, January 5, 2001 at 1098, col. 3 (emphasis added). In the instant case, the Examiner has provided generalized statements that utilities asserted for the polypeptide SEQ ID NO:35 are not substantial because "the specification does not disclose any specific diseases associated with altered levels or forms of the protein. There is no disclosure, for example, of any symptoms associated with such a disease." *See* Paper No. 9, page 5, lines 17-19. Contrary to the Examiner's allegation, Applicants have identified specific diseases that could be diagnosed by altered levels of the protein, namely brain, bladder, ovarian and skin cancer. *See* specification at page 11, line 30 to page 12, line 2. Nevertheless, while the Examiner has acknowledged that Applicants have asserted utilities in the specification, the utilities are dismissed as being insubstantial or non-specific. Importantly, insufficient explanation setting forth the reasoning or factual support used in reaching this conclusion has been given. For

instance, it is unclear why disease symptoms must be described to fulfill the requirements of 35 U.S.C. § 101.

The M.P.E.P. defines a "substantial utility" as a utility with real world use. *See* M.P.E.P. § 2107.01. The M.P.E.P. further states in the same section, "An assay that measures the presence of a material which has a stated correlation to a predisposition to the onset of a particular disease condition would also define a 'real world' context of use ...". Applicants assert in the specification, "aberrant expression of this gene product in tissues - particularly adult tissues - may correlate with patterns of abnormal cellular proliferation, such as found in many various cancers." *See* specification at page 12, lines 25-27. Thus, combined with the specific types of cancer Applicants disclose in the specification, Applicants submit that their asserted utility is substantial.

Applicants have previously asserted that SEQ ID NO:35 shares sequence homology with the tumor suppressor gene product, deleted in bladder cancer critical region 1 (DBCCR1). DBCCR1 encodes a putative 761 amino acid protein which when deleted or when its promoter is hypermethylated (silenced), results in transitional cell carcinoma of the bladder. *See* Habuchi *et al.* (2001) and Nishiyama *et al.* (2001) submitted herewith as Exhibits A & B respectively. According to the Utility Examination Guidelines, "when a patent application ... bases the assertion on homology to existing nucleic acids or proteins having an accepted utility, the asserted utility must be accepted by the Examiner unless the Office has sufficient evidence or sound scientific reasoning to rebut such an assertion." In the instant rejection, the Examiner has not provided such reasoning or evidence.

In support of the asserted utility of Secreted Protein HCE3C63 as a tumor suppressor, *i.e.*, a tumor diagnostic, Applicants submit Secreted Protein HCE3C63 shares several functional domains with DBCCR1. As evidenced in Exhibit C, both proteins have several phosphorylation sites (shaded), N-myristylation sites (in bold), N-glycosylation site (underlined) and a cysteine-rich region (boxed) in common over the length of their entire amino acid sequence. *See* Alignment submitted herewith as Exhibit C. *See* also Exhibits D & E (PROSITE analysis of SEQ ID NO:35 and DBCCR1 respectively; http://us.expasy.org/prosite). Since conserved protein domains and motifs represent evolutionary important structures, proteins sharing such conserved sequences likely have similar tertiary structures and possess similar functions. Applicants respectfully submit that one of skill in the art would conclude that Secreted Protein HCEC63 functions as a tumor suppressor like DBCCR1. Applicants submit that based on the foregoing evidence, it is more

likely than not that one skilled in the art would consider Applicants' asserted utility to be substantial.

In view of the above arguments, Applicants have provided evidence and reasoning which supports the Applicants' assertion of utility. In particular, Applicants have provided evidence that the polypeptides and/or antibodies raised against the polypeptide of the instant application are useful as a cancer diagnostic. This utility asserted in the specification for Secreted Protein HCE3C63 (SEQ ID NO:35) is indeed specific, substantial and credible. Accordingly, Applicants respectfully submit that the rejection of claims 25-46 under 35 U.S.C. § 101 has been obviated. Therefore, Applicants respectfully request that the rejection be reconsidered and withdrawn.

For the reasons discussed above in response to the rejection under 35 U.S.C. § 101, the claimed invention is supported by a specific, substantial and credible asserted utility. The Examiner "should not impose a 35 U.S.C. § 112, first paragraph, rejection grounded on a 'lack of utility' basis unless a 35 U.S.C. §101 rejection is proper." M.P.E.P. § 2107 (IV) at 2100-36. Therefore, because the claimed invention complies with the utility requirement of 35 U.S.C. § 101, the rejections under 35 U.S.C. § 112, first paragraph, based on the alleged lack of utility of the claimed invention, should be withdrawn. Accordingly, Applicants respectfully request that the rejection under 35 U.S.C. § 112, first paragraph, be reconsidered and withdrawn.

II. Rejection of Claims Under 35 U.S.C. § 112, First Paragraph

A. Written Description of Claims 15 and 25-46

Claims 15 and 25-46 have been rejected under 35 U.S.C. § 112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. *See* Paper No. 9, page 6, lines 13-15.

Applicants respectfully disagree and traverse.

Preliminarily, Applicants point out that claim 15 has been canceled, thus rendering the rejection to claim 15 moot. Applicants respectfully request withdrawal of the rejection to claim 15.

As the Examiner has noted, Applicants disclose in the specification that the HCE3C63 cDNA contained in ATCC Deposit No. PTA-909 was deposited at the ATCC on

November 2, 1999 on page 4 of the specification. The specification clearly discloses that ATCC Deposit No. PTA-909 has been deposited under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure with the following International Depository Authority: American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, Virginia 20110-2209, U.S.A. (*See* page 4, lines 5-13). The Applicants respectfully submit that the specification is in compliance with 37 C.F.R. §§ 1.801-1.809.

Nevertheless, Applicants submit herewith a declaration regarding availability of the deposit made in connection with the present application under the Budapest Treaty.

As attorney for the above-identified Applicants in the above-identified patent application, I hereby declare and state that:

Human Genome Sciences, Inc., the assignee of the present application, has deposited biological material under the terms of the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for the Purposes of Patent Procedure with the following International Depository Authority: American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, Virginia 20110-2209. The deposit was made on November 2, 1999, and given ATCC Accession Number PTA-909. In accordance with M.P.E.P. § 2410.01 and 37 C.F.R. § 1.808, assurance is hereby given that all restrictions on the availability to the public of ATCC Accession Number PTA-909 will be irrevocably removed upon the grant of a patent based on the instant application, except as permitted under 37 C.F.R. § 1.808(b). A partially redacted copy of the ATCC Deposit Receipt for Accession Number PTA-909 is enclosed herewith as Exhibit F.

In view of the above, Applicants submit that the rejection under 35 U.S.C. § 112, first paragraph, has been obviated. Accordingly, Applicants respectfully request that this rejection be reconsidered and withdrawn.

B. Written Description of Claims 37-46

Claims 37-46 have been rejected under 35 U.S.C. § 112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. Specifically, claims 37 and 42 are directed to an isolated protein comprising an amino acid sequence at least 90% identical to the reference protein.

Preliminarily, Applicants point out that claims 37 and 42 have amended to recite the phrase "consisting of" rather than "comprising." Applicants assert that the claims, as amended, are fully enabled since one of ordinary skill in the art can make and/or use every single polypeptide variant embraced by the claims. Thus, claims 37-46 fully meet the enablement requirement of 35 U.S.C. § 112, first paragraph.

Nevertheless, the test for the written description requirement is whether one skilled in the art could reasonably conclude that the inventor has possession of the claimed invention in the specification as filed. *Vas-Cath Inc. v. Mahurkar*, 935 F.2d 1555, 1563, 19 U.S.P.Q.2d 1111, 1116 (Fed. Cir. 1991); M.P.E.P. § 2163.02.

The Federal Circuit recently re-emphasized the well-settled principle of law that "[t]he written description requirement does not require the applicant 'to describe exactly the subject matter claimed, [instead] the description must clearly allow persons of ordinary skill in the art to recognize that [they] invented what is claimed,'" *Union Oil Co. v. Atlantic Richfield Co.*, 208 F.3d 989, 54 U.S.P.Q.2d 1227 (Fed. Cir. 2000), hereinafter referred to as "*Unocal.*" While the applicant must "blaze marks on trees," rather than "simply [provide] the public with a forest of trees," an Applicant is not required to explicitly describe each of the trees in the forest. *See Unocal*, 208 F.3d at 1000. *See also* M.P.E.P. § 2163.02 ("The subject matter of the claim need not be described literally (*i.e.*, using the same terms or *in haec verba*) in order for the disclosure to satisfy the description requirement."). The Court emphasized the importance of what the person of ordinary skill in the art would understand from reading the specification, rather than whether the specific embodiments had been explicitly described or exemplified. Indeed, as the court noted, "the issue is whether one of skill in the art could derive the claimed ranges from the patent's disclosure." *Unocal*, 208 F.3d at 1001 (emphasis added).

In an analysis of written description under 35 U.S.C. § 112, first paragraph, the Examiner bears the initial burden of presenting a *prima facie* case of unpatentability. This

burden is discharged if the Examiner can present evidence or reasons why one skilled in the art would *not* reasonably conclude that Applicants possessed the subject matter as of the priority date of the present application. *In re Wertheim*, 541 F.2d 257, 262, 191 USPQ2d 90, 96 (C.C.P.A. 1976); M.P.E.P. § 2163.04.

Applicants submit that one skilled in the art would reasonably conclude that Applicants had possession of the polypeptides encompassed by the rejected claims in the present application as filed. Applicants further submit that the Examiner has underestimated both the teaching of the present application and the level of skill in the art on the priority date of the present application.

Applicants recognize that the Examiner is in part relying on language regarding a "representative number" of a claimed genus set forth in *Regents of the University of California v. Eli Lilly & Co.*, (119 F.3d 1559, 1569, 43 U.S.P.Q.2d 1398, 1406 (Fed. Cir. 1997)) (hereinafter "*Eli Lilly*") and incorporated into the Guidelines for Examination of Patent Applications Under the 35 U.S.C. § 112, ¶ 1 "Written Description" Requirement ("Guidelines"), when reciting the procedures followed in analyzing whether the description requirement for each of the claims at issue is satisfied. The central issue in *Eli Lilly* involved claims to all mammalian cDNAs encoding insulin, which were supported in the specification only by the nucleotide sequence for the rat insulin gene. The Federal Circuit found the claims to human insulin lacked written description because the claims defined only a result or function. The court held that a result or function would satisfy the written description requirement *only if* correlated to a description of structural features of the claimed invention. According to the court, a sufficient written description must allow the skilled artisan to "visualize or recognize the identity of the members of the genus." *Id.*

In addition, the court held in *Eli Lilly* that a description of a genus of cDNAs may be achieved by reciting a representative number of cDNAs, defined by nucleotide sequence, falling within the scope of the genus or by reciting structural features common to a substantial portion of the members of the genus. *Eli Lilly*, 119 F.3d 1559, 1569 (Fed. Cir. 1997). Therefore, it logically follows that claims to polypeptides encoded by cDNAs may also be satisfied by providing sequences of a representative number of polypeptides which fall within the scope of the genus *or* by providing a recitation of structural features common to a substantial portion of the members of the genus.

Applicants assert that, in the instant case, the second test set forth in *Eli Lilly* has been satisfied because Applicants' description of the reference polypeptide sequence, SEQ ID

NO:35, provides one skilled in the art with the necessary structural features common to a substantial portion of the members of the genus. Applicants further point out that the recitation of the structural features of the reference protein is a recitation of the structural features common to the members of the claimed genus because the proteins included within the claimed genus will have at least 90% (or at least 95%) of the amino acids of their amino acid sequence primary structure in common to the reference polypeptide of SEQ ID NO:35. Indeed, nothing more than a basic knowledge of the genetic code and what is described in the specification would be required for the skilled artisan to identify every single one of the polypeptides consisting of 90% or 95% identical to the amino acid sequence of SEQ ID NO:35. Clearly, such knowledge is well within what is expected of the skilled artisan. Therefore, in accord with *Eli Lilly,* the specification clearly conveys that Applicants were in possession of the claimed invention on the priority date of the instant application.

In view of the above, Applicants respectfully assert that the Examiner has failed to meet the required burden in presenting evidence or reasons why those skilled in the art would not recognize the claimed invention from the disclosure. Moreover, the specification conveys with reasonable clarity that Applicants were in possession of the claimed invention. Therefore, Applicants submit that the claims fully meet the written description requirements of 35 U.S.C. § 112, first paragraph, and respectfully request that the Examiner's rejection of claims 35-55 under 35 U.S.C. § 112, first paragraph, be reconsidered and withdrawn.

III. Rejection of Claim 15 Under 35 U.S.C. § 112, Second Paragraph

Claim 15 has been rejected under 35 U.S.C. § 112, second paragraph as allegedly being indefinite. *See* Paper No. 9, page 8, section 7.

Applicants point out that claim 15 has been canceled, thus rendering the rejection to said claims moot. Applicants respectfully request withdrawal of the rejection to claim 15 under 35 U.S.C. § 112, second paragraph.

Conclusion

Applicants respectfully request the amendments and remarks of the present response be entered and made of record in the present application. In view of the foregoing amendment and remarks, Applicants believe they have fully addressed the Examiner's concerns and that this application is now in condition for allowance. An early notice to that

effect is urged. The Examiner is invited to call the undersigned at the phone number provided below if any further action by Applicant would expedite the allowance of this application.

Applicants believe that there are no fees due in connection with the filing of this paper. However, should a fee be due, please charge the fees to our Deposit Account No. 08-3425. If a fee is required for an extension of time under 37 C.F.R. § 1.136, such an extension is requested and the appropriate fee should also be charged to our Deposit Account.

Respectfully submitted,

Date: October 1, 2003

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SHORT REPORTS

Hypermethylation at 9q32-33 tumour suppressor region is age-related in normal urothelium and an early and frequent alteration in bladder cancer

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Transcriptional silencing by CpG island hypermethylation of gene regulatory regions is one mechanism for inactivation of tumour suppressor genes. Chromosome 9q deletion is frequently found in transitional cell carcinoma (TCC) of the bladder and upper urinary tract and one of the putative tumour suppressor loci has been mapped to 9q32-33. A gene designated as *DBCCR1* was identified in the candidate region and its mRNA expression is thought to be suppressed by hypermethylation. To understand the role of hypermethylation in TCC, we evaluated the methylation status of 20 CpG sites of the *DBCCR1* 5′-CpG island region in a total of 69 tumours from 45 patients, 21 normal urothelial specimens, and six bladder cancer cell lines. Aberrant hypermethylation levels were found in 36 (52%) of 69 tumours without any association with tumour grade or stage. Methylation was weakly detected in the normal urothelium in association with ageing. Although recurrent tumours tended to have higher methylation levels than the initial tumours, the methylation pattern was mostly maintained between multifocal TCCs in individual patients. The results suggest that hypermethylation of the *DBCCR1* region is one of the earliest alterations in the development of TCCs and there may be an age-related hypermethylation-based field defect in normal urothelium. Methylator or methylation-resistant phenotype seems to be maintained during multifocal development or recurrence of most TCCs. *Oncogene* (2001) **20,** 531–537.

Keywords: bladder cancer; methylation; *DBCCR1*; chromosome 9q

Mammalian DNA methylation is a covalent modification of cytosine residues, predominantly at CpG dinucleotides, and the methylation pattern in somatic cells is generally well maintained with normal cell division (Ceder and Razin, 1990). In contrast, isolated CpG dinucleotides in bulk chromatin are often methylated, whereas cytosine residues in CpG islands are unmethylated and such islands are mostly located in the promoters or coding regions of genes (Cross and Bird, 1995). Accumulating evidence shows that aberrant hypermethylation at CpG dinucleotides in normally unmethylated CpG islands of gene promoter regions may repress gene expression. In fact, transcriptional silencing of tumour suppressor genes, such as the RB1 gene (Sakai *et al.*, 1991), the VHL gene (Herman *et al.*, 1994) and the p16 gene (Merlo *et al.*, 1995; Gonzalez-Zulueta *et al.*, 1995; Herman *et al.*, 1995), by aberrant hypermethylation of regulatory sequences has been reported. Hypermethylation of CpG dinucleotides in CpG islands may constitute an alternative mechanism to intragenic mutations or gene deletions for the inactivation of tumour suppressor genes (Cross *et al.*, 1995; Jones and Laird, 1999).

Transitional cell carcinomas (TCCs) of the bladder, ureter and renal pelvis are among the most common human cancers and have a few distinct characteristics (Messing and Catalona, 1998). The majority of TCCs are low-grade non-invasive tumours which occur often in heterotropic urothelium but progress infrequently to the invasive phenotype. In contrast, muscle-invasive TCCs often metastasize to other organs and have a poor prognosis, and is therefore considered a systemic disease. In addition, TCCs are characterized by common synchronous and/or metachronous multifocal development throughout the urothelium. Recent molecular genetic studies have shown that loss of heterozygosity (LOH) on chromosome 9q is one of the most frequent genetic alterations and is found consistently in all stages and grades of TCC, indicating the presence of an important tumour suppressor gene(s) on 9q (Knowles *et al.*, 1994; Habuchi *et al.*, 1995). Detailed LOH studies on 9q have indicated that there are multiple tumour suppressor loci for TCC (Habuchi *et al.*, 1995, 1997). One of the putative tumour suppressor loci on 9q was mapped at 9q32-33 (DBC1) and a gene designated as *DBCCR1* (deleted in bladder cancer chromosomal region candidate 1) was identified (Habuchi *et al.*, 1997, 1998). Although it is unclear if *DBCCR1* is a real tumour suppressor gene, exon 1 is very rich in CpG sites and conforms to the criteria for

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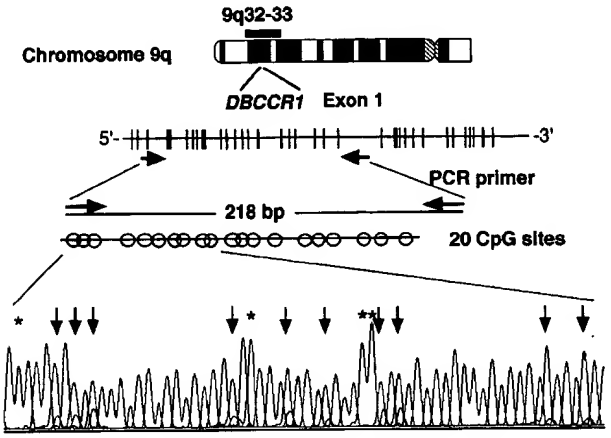
a



b

Name	CpG Site 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20	MS	DBCCR1 Expression
5637	●●●●●●●●●●●●●●●●●●●●	62	No
T24	●●●●●●●●●●●●●●●●●●●●	80	No
HT1197	○○○○○○○○●●●●●○●○○○●	21	No
RT112	○○○○○○○○○○○○●○○●○○○○	6	Yes
253J	●●●●●●●●●●●●●●●●○●●●	40	Yes
UMUC3	●●●●●●●●·●●●○●●●●●	47	Yes

Figure 1 The analysis of CpG methylation status of the *DBCCR1* 5′-region at the 9q32-33 candidate tumour suppressor locus. (**a**) A map of *DBCCR1* exon 1 and location of primers for PCR and sequencing (horizontal arrows) with a representative direct sequencing electrophoretogram from a tumour with heavy hypermethylation. The CpG sites are plotted in vertical lines. Vertical arrows indicate methylated CpG sites. * = thymines modified by bisulfite treatment from cytosines at non-CpG sites. Genomic DNA extracted from the tumour and normal samples was treated with sodium bisulfite as described previously (Frommer *et al.*, 1992). The selected 218 bp region containing 20 CpG sites of the exon 1 of the *DBCCR1* was amplified from bisulfite-modified DNA by PCR. PCR primer sequences specific for bisulfite-converted DNA were 5′-GGATTTTA(T/C) GGTTGTAAATTGATTG (forward, modified from nt 11–36 by GenBank Accession No. AF027734) and 5′-CCTAACAACC-TAACTCATACTCAAC (reverse, modified from nt 206–230). DNA sequencing was performed by ABI PRISM™377 (PE Applied Biosystems). Since unmethylated cytosines appeared as thymines, whereas methylated cytosines remained as cytosines after bisulfite modification, methylation status was determined by comparing the intensity of sequencing electropherogram of cytosine with that of thymine at each CpG site. When the intensity of cytosine was less than 20% that of thymine, the CpG site was defined as having 'no methylation' and was given a methylation score of '0'. When the intensity of cytosine was from 20–50% that of thymine, the site was defined as having 'minor methylation' and given a score of '1'. When the intensity of cytosine was from 50–200% that of thymine, the site was defined as having 'partial methylation' and given a score of '2'. When the intensity of cytosine was more than 200% that of thymine, the site was defined as having 'complete methylation' and given a score of '4'. Since the intensity of the sequencing electropherogram varies according to surrounding nucleotide sequences and is not absolutely quantitative, our evaluation for methylation status at each CpG site was considered as semi-quantitative. (**b**) Methylation profiles of 20 CpG sites in the *DBCCR1* 5′-region in six bladder cancer cell lines. *DBCCR1* mRNA expression was determined by RT–PCR analysis as described elsewhere (Habuchi *et al.*, 1998), with a minor modification. MS = total methylation score. White circles = No methylation, scored as '0'. Dotted circles = minor methylation, scored as '1'. Striated circles = partial methylation, scored as '2'. Black circles = complete methylation, scored as '4'. *denotes a mutation or polymorphism at this CpG site (G to T), resulting in the removal of the CpG sequence

a CpG island (Habuchi *et al.*, 1998). Furthermore, its mRNA expression appears to be suppressed by hypermethylation of the 5′-region, which is frequently found in TCCs *in vivo* and *in vitro* (Habuchi *et al.*, 1998). However, the biological significance of the hypermethylation of the *DBCCR1* 5′-region is unclear. It also remains unknown whether this is an early or late event in the development of TCCs and whether hypermethylation is a tumour-specific alteration.

In the present study, we have tried to delineate the timing and biological significance of hypermethylation of the *DBCCR1* 5′-region at the 9q32-33 candidate tumour suppressor locus. A total of 69 urothelial TCC specimens from 45 patients, 21 normal urothelial specimens, and six bladder cancer cell lines were included in this study.

Exon 1 of *DBCCR1* is non-coding and has been shown to conform to the criteria of the CpG island (Habuchi *et al.*, 1998; Gardiner-Garden and Frommer, 1987). The selected 218 bp region containing 21 CpG sites of the exon 1 was amplified from bisulfite-modified DNA by PCR and directly sequenced (Figure 1a). The methylation status of the region and the presence of its mRNA expression in the six bladder cancer cell lines are summarized in Figure 1b). RT–PCR analysis of *DBCCR1* mRNA expression revealed that cell lines RT112, 253J and UMUC3 showed expression of *DBCCR1* mRNA, whereas there was no expression in cell lines T24, 5637 and HT1197. The result was in accord with the previous study (Habuchi *et al.*, 1998). Sequencing of bisulfite-treated genomic DNA showed partial to complete methylation at all the 20 CpG sites in the T24 and 5637 cell lines, and partial to complete methylation at more than the 16 CpG sites in the 253J and UMUC3 cell lines (Figure 1b). Therefore, there seemed to be no clear association between the extent of methylation and the mRNA expression level. Furthermore, there was no specific site for methylation that was associated with loss of mRNA expression among the 20 CpG sites.

Because it is unknown whether hypermethylation in this region is urothelial cancer specific or whether it is present even in normal urothelium, we examined the methylation status of the *DBCCR1* 5′-region in the normal urothelium from 21 subjects ranging in age from six to 80 years. Methylation of several of the 20 CpG sites seemed to be correlated with ageing. By scoring the methylation level at each CpG site based on the ratio of electrophoretogram intensity of cytosine and thymine as described, the methylation score was plotted against the age of the subjects (Figure 2). A regression analysis of all normal samples gave a linear fit with a correlation coefficient of 0.389 (R squared),

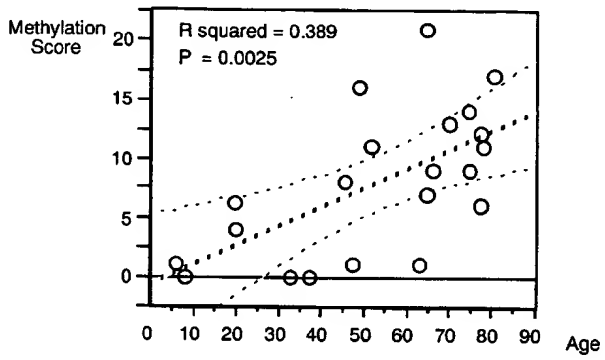


Figure 2 Ageing and methylation of the *DBCCR1* 5′-region. The methylation score from normal urothelium calculated by the cytosine to thymine ratio of direct sequencing of bisulfite-modified DNA is plotted against the age of 21 non-cancer urologic patients or healthy kidney donors. Normal transitional cell epithelium was peeled away from submucosal connective tissue and subjected to DNA extraction. A regression analysis of the 21 samples gave a linear fit and was statistically significant ($P=0.0025$)

which is considered to be statistically significant ($P=0.0025$, Figure 2). There was a variation in the degree of hypermethylation between each CpG site, since some CpG sites showed frequent partial methylation (data not shown), whereas other sites were almost completely free from methylation.

Since the chronological tracing of genetic and epigenetic alterations is possible by studying synchronous or metachronous multifocal TCCs (Takahashi *et al*., 1998), we then tested the methylation status of the *DBCCR1* 5′-region in 44 multifocal tumours from 21 patients. All these tumours were tested for microsatellite alterations in the previous study (Takahashi *et al*., 1998). Methylation profiles of multifocal tumours with concordant microsatellite alterations are shown in Figure 3a and those of tumours with discordant microsatellite alterations in Figure 3b. Partial or complete methylation at eight or more CpG sites was found in 13 (48%) of 27 tumours in the concordant group and four (22%) of 18 tumours in the disconcordant group ($P=0.073$, Fisher's exact test). Next, the methylation pattern was compared between tumours in each patient with multifocal tumours. Five (24%) of the 21 patients showed a discordant methylation pattern at four or more CpG sites tested. A discordant methylation pattern at four or more sites was found in one (17%) of six patients with synchronous multifocal tumours and four (27%) of 15 patients with metachronous multifocal tumours ($P>0.99$, Fisher's exact test). These results indicated that methylation patterns were maintained in the majority of CpG sites in most multifocal tumours. In metachronous tumours, recurrent tumours seemed to have a tendency to harbour higher methylation status than the initial tumours tested. Of the 18 evaluable pairs of metachronous tumours, 13 (72%) recurrent tumours had higher methylation scores than the initial tumours, whereas only four recurrent tumours had lower methylation scores. This deviation toward the higher methylation scores was statistically significant ($P=0.0395$, Wilcoxon signed rank test).

Recent studies have suggested that hypermethylation of the CpG island of some genes is associated with tumour progression (Issa *et al*., 1997; Toyota *et al*., 1999; Maesawa *et al*., 1996) while that of other genes is an early event in the tumorigenesis (Sakai *et al*., 1991; Herman *et al*., 1994; Issa *et al*., 1994; Ahuja *et al*., 1998). To determine whether hypermethylation of the *DBCCR1* 5′-region is associated with tumour progression, we examined 24 urothelial cancers consisting of 10 low grade (grade 1 or 2)–low stage (Ta or T1) tumours and 14 high grade (grade 3)–high stage (pT2 or more) tumours (data not shown). The difference in the methylation score between the two groups was not statistically significant ($P=0.953$, the Mann–Whitney *U*-test). Next, by combining the data of the multifocal tumour cases, the methylation scores of all the tested tumours were plotted with box-and-whiskers according to tumour grade and tumour stage (Figure 4). The statistical analysis showed no significant difference in the methylation score between any combination of groups divided by tumour grade or stage ($P=0.673$ for tumour grade, $P=0.154$ for tumour grade, the Kruskal-Wallis test). Since most urothelial cancer patients are over 50 years old (only three (7%) of 45 TCC patients in this study were under 50 years old), we calculated the normal level of methylation in men over 50 years old. Of the 21 normal urothelium, 12 subjects met this criteria and the mean$\pm$s.d. (standard deviation) of the methylation score was 10.9 ± 5.2. If the cut-off value was placed at mean$+2\times$s.d., the methylation scores of 22 or more might be considered to be abnormal. With this criteria, 36 (52%) of 69 tumours in total were judged to have an abnormal hypermethylation level of the *DBCCR1* 5′-region. Abnormal hypermethylation was found in seven (54%) of 13 grade 1, 21 (50%) of 42 grade 2, and eight (57%) of 14 grade 3 tumours ($P>0.05$, Chi-square test). As for tumour stage, the abnormal hypermethylation was found in 12 (39%) of 31 pTa, 13 (72%) of 18 pT1 and nine (53%) of 17 pT2-4 or metastatic tumours ($P>0.05$, Chi-square test).

The present study shows that the *DBCCR1* 5′-region is a frequent (about 50% or more) target of aberrant hypermethylation in TCCs *in vivo*. However, there was no clear relationship between *DBCCR1* mRNA expression and the hypermethylation pattern. In the previous study, *de novo* re-expression of *DBCCR1* mRNA was found in T24 and 5637 cell lines after treatment with the demethylating agent 5-aza-2′-deoxycytidine (Habuchi *et al*., 1998), indicating that hypermethylation-based silencing is involved in the *DBCCR1* mRNA silencing. Because the CpG island tested in this study is located in the 3′-region of the transcription start site (Habuchi *et al*., 1998) and the CpG-rich region extends to 5′ (MA Knowles, unpublished data), it is probable that there are other CpG sites or a CpG-rich region which are critical for the regulation of mRNA expression. Since gene silencing by CpG methylation seems to be somewhat dependent

on the CpG methylation density (Hsieh, 1994) and methylation status in the promoter region which is not transcribed (Jones, 1999), more extensive methylation mapping would reveal a clearer relationship between the methylation level of the *DBCCR1* 5′-region and its mRNA expression.

Although it has not yet been clarified whether *DBCCR1* is a real tumour suppressor, several lines of evidence show that the region containing *DBCCR1* is a strong candidate tumour suppressor locus (Habuchi, 1997; Nishiyama *et al*., 1999; van Tilborg *et al*., 1999). The frequent hypermethylation in TCCs and the presence of hypermethylation in the normal urothelium suggests that the region is an easy and frequent target of random CpG hypermethylation. Such random CpG hypermethylation may give rise to reduced expression of genes in this region and increased heterochromatinization, leading to clonal selection of transitional cells with abnormal hypermethylation, as observed in the present study (Jones, 1996, 1999). It would be interesting to know if the frequent hypermethylation in the region has any biological relationship with frequent occurrence of LOH on 9q in TCCs. LOH on 9q coupled with hypermethylation-based gene silencing of the remaining allele may be one of the major mechanisms for the inactivation of a tumour suppressor gene at the 9q32-33 locus. While hypermethylation of the CpG island of some genes may be associated with tumour progression (Issa *et al*., 1997; Toyota *et al*., 1999; Maesawa *et al*., 1996), our data suggests that the hypermethylation of the *DBCCR1* 5′-region is an early event in the development of TCCs. It remains to be seen whether the early occurrence of hypermethylation at this locus is related to the fact that the LOH at 9q32-33 is one of the earliest events in the development of TCC.

The presence of the age-related methylation in the normal urotherlium supports the conclusion that

a

Tumor	CpG Site 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20	MS	Dx	G	S	Microsatellite Alteration
1-2 MT		21	1	2	a	9p-S, 9q-L, 11p-L
1-4 MT		17	1	1	a	9p-S, 9q-L, 11p-L
2-4 MT		22	1	2	a	4pq-L, 9q-L, 17p-L
2-6 MT		45	2	2	a	4pq-L, 9q-L, 17p-L
3-1 MT		32	1	2	1	9p-S, 11p-L
3-2 MT		50	1	2	1	9p-S, 11p-L
7-1 MT		22	1	2	a	4q-L, 9q-L, 11p-L
7-2 MT		38	1	2	a	4q-L, 9q-L, 11p-L
9-1 MT		18	1	2	a	4pq-L, 9p-S, 9q-L, 11p-L
9-4 MT		13	1	1	a	4pq-L, 9p-S, 9q-L, 11p-L
9-7 MT		31	1	1	a	4pq-L, 9p-S, 9q-L, 11p-L
10-1 MT		3	2	2	1	11p-L, 17p-L
10-2 MT		11	1	2	1	11p-L, 17p-L
15-1 MT		51	1	2	1	11p-L, 17p-L
15-2 MT		70	1	2	1	11p-L, 17p-L
18-1 MT		17	1	1	a	4p-L, 9q-L
18-2 MT		21	1	1	a	4p-L, 9q-L
19-1 ST		6	1	1	a	9p-S
19-2 ST		25	1	1	n	9p-S
26-1 ST		37	1	2	1	2q-S, 8p-L, 17p-L
26-2 ST		47	1	2	1	2q-S, 8p-L, 17p-L
27-1 ST		22	1	2	1	8p-S
27-2 ST		22	1	2	1	8p-S
31-1 ST		25	1	2	a	9q-L
31-3 ST		21	1	2	a	9q-L
32-1 ST		43	1	2	a	4p-L, 9p-S, 9q-S
32-2 ST		44	1	2	a	4p-L, 9p-S, 9q-S

hypermethylation in this region is an early event in the development of TCC. Such age-related progressive methylation has been reported in the CpG islands of a few candidate tumour suppressor genes – *Estrogen receptor* (Issa *et al.*, 1994), *N33* (Li *et al.*, 1998; Ahuja *et al.*, 1998), an imprinted *IGF2* (Issa *et al.*, 1996) and a proto-oncogene c-*fos* (Choi *et al.*, 1996). In colorectal mucosal cells, age-related methylation seems to be both gene-specific and tissue-specific (Ahuja *et al.*, 1998). Although the hypermethylation level of normal urothelium was much lower than that of TCCs, the weak age-related methylation could be the basis of field-cancerization of the urothelium and may be related to the fact that most TCCs are found in the aged population (Messing and Catalona, 1998). Furthermore, evidence for the methylation-based field cancerization of the normal urothelium in bladder cancer patients has been observed by Muto *et al.* (2000). Because of a considerable difference in the extent of methylation of normal urothelium even in age-matched subjects (Figure 2), it would be interesting to know whether it is caused by a difference in exposure to carcinogens or a difference in genetic or endogenous factors to maintain the integrity of the CpG methylation pattern. Furthermore, it is critical to ascertain whether the hypermethylated normal urothelium is more susceptible to development of TCC. If this is the case, drugs to inhibit *de novo* DNA methylation could be useful for preventing the development of TCC in a subset of individuals with methylator phenotype.

Our results indicate that the methylation pattern of most multifocal tumours is maintained in each patient, irrespective of other microsatellite alterations or LOH. TCCs with methylator phenotype or TCCs with methylation-resistant phenotype tended to maintain their phenotype during the multifocal development or heterotopic recurrence. Furthermore, a striking difference in methylation levels according to each tumour and each cell line suggests that there is a phenotype of extensive CpG methylation. The results warrant further study to clarify the cause of this CpG methylator phenotype, such as altered regulation of DNA methyltransferase genes (Issa, 1999).

In conclusion, hypermethylation of the *DBCCR1* 5′-region at 9q32-33 is frequent and one of the earliest alterations in the development of TCCs. There is age-

b

Tumor	CpG Site 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20	MS	Dx	G	S	Microsatellite Alteration
5-1 MT		16	1	2	1	2q-L, 4pq-L, 8p-L, 9pq-L, 11p-L
5-2 MT		17	1	2	a	2q-L, 8p-L, 9pq-L, 11p-L
16-1 MT		1	2	2	a	9p-S, 9q-L, 17p-L
16-2 MT		8	1	2	n	9p-S, 9q-L, 11p-L
16-3 MT		9	1	2	n	9p-S, 9q-L, 11p-L, 17p-L
21-1 MT		0	1	2	1	8p-L, 9q-S, 17p-L
21-2 MT		2	1	2	1	2q-L, 9q-S
21-4 MT		0	1	2	a	2q-L, 9q-S
22-1 MT		13	2	2	2	9q- L, 17p-L
22-2 MT		22	1	3	1	2q-L, 4p-L, 9q-L, 11p-L, 17p-L
23-1 MT		28	2	2	3	8p-L, 9q-L
23-2 MT		9	1	2	a	4q-L, 8p-L, 9q-L, 17p-L
6-1 MT		3	2	2	a	9p-SL, 9q-L
6-2 MT		10	1	2	a	9q-L
30-1 MT		27	1	2	a	8p-L, 9pq-L, 17p-L
30-2 MT		19	1	2	a	9pq-L
35-1 ST		15	2	2	3	2q-L1, 9q-L
35-2 ST		27	1	2	1	2q-L2, 9p-L, 9q-L

Figure 3 Methylation profiles of 20 CpG sites in the *DBCCR1* 5′-region in synchronous or metachronous multifocal TCCs. (**a**) Multifocal tumour cases with concordant microsatellite alterations. (**b**) Multifocal tumour cases with discordant microsatellite alterations. In the tumour column, 'MT' indicates metachronous multifocal tumours and 'ST' indicates synchronous multifocal tumours. MS = total methylation score. Dx = primary site of each tumour (1 = primary bladder tumour, 2 = primary renal pelvic or ureteral tumour). G = tumour grade classified according to the WHO criteria (Mostofi *et al.*, 1973). S = tumour stage (T-category) by TNM classification (UICC, 1992). Annotations for circles are the same as those shown in Figure 1. In the microsatellite alteration column, -S and -L represent microsatellite shift (instability) and loss of heterozygosity, respectively. The results for microsatellite analysis were published previously (Takahashi *et al.*, 1998). In comparing the methylation status at each CpG site between multifocal tumours in a single patient, the methylation status was defined as 'discordant' when there was a 2-degree difference (i.e.; from 'minor methylation' to 'complete methylation', from 'no methylation' to 'partial methylation', or *vice versa*) between two tumours

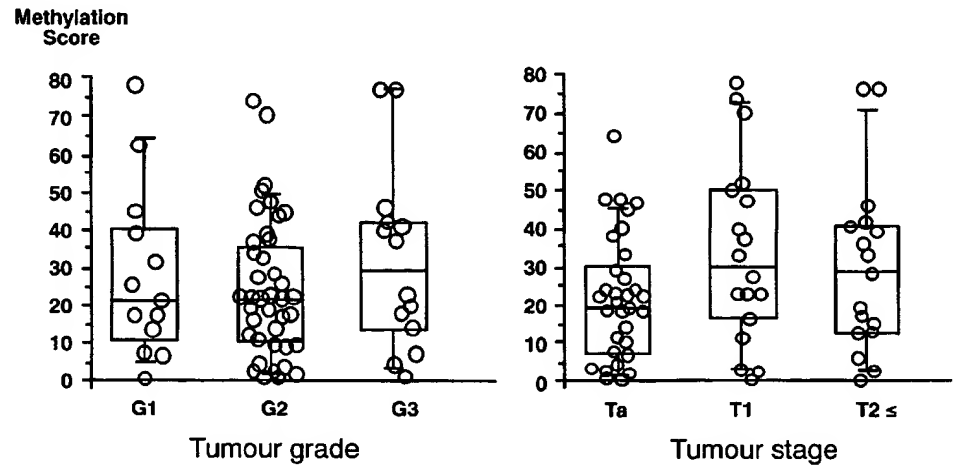


Figure 4 Tumour grade and stage and methylation level of the *DBCCR1* 5′-region. The total methylation score of 20 CpG sites is plotted against tumour grade and tumour stage. Boxes, lines and whiskers represent 25th to 75th percentile, median and 11th to 90th percentile values. No significant difference was found in tumour grade and tumour stage ($P=0.673$ for tumour grade, $P=0.154$ for tumour grade, the Kruskal-Wallis test)

related hypermethylation in the normal urothelium, which could be the basis of a field defect in the aged urothelium. Methylator or methylation-resistant phenotype seems to be maintained during multifocal development or recurrence of most TCCs.

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Negative regulation of G_1/S transition by the candidate bladder tumour suppressor gene *DBCCR1*

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Deletion of all or part of chromosome 9q is the most common genetic alteration in all stages and grades of bladder cancer. *DBCCR1* (*d*eleted in *b*ladder *c*ancer *c*hromosome *r*egion candidate *1*) maps to the chromosome region 9q32-33, a candidate tumour suppressor locus for bladder cancer. Although no mutations of *DBCCR1* have been detected in bladder tumours, expression of *DBCCR1* is silenced by promoter hypermethylation in 50% of bladder cancer cell lines analysed. Here we sought to provide functional evidence to authenticate *DBCCR1* as a tumour suppressor using gene-transfer methods. Exogenous expression of DBCCR1 protein or an HA epitope-tagged fusion protein, HA-DBCCR1 in NIH3T3 cells and human bladder tumour cell lines resulted in suppression of proliferation. Cell cycle analyses in NIH3T3 cells revealed that DBCCR1-mediated growth inhibition was due to an increase in the number of cells in the G_1 phase of the cell cycle. The levels of apoptosis were not altered. These results demonstrate a role for *DBCCR1* in cell cycle control, thereby supporting the hypothesis that this is the tumour suppressor gene targeted by 9q32-33 deletion in bladder cancer. *Oncogene* (2001) **20,** 2956–2964.

Keywords: transitional cell carcinoma; *DBCCR1*; cell cycle

Introduction

Transitional cell carcinoma (TCC) of the urothelium of the bladder, ureter and renal pelvis is amongst the commonest of human cancers with 12 000 new cases in the UK and 54 000 in the US per annum (Parker *et al.*, 1997; Office for National Statistics, 1996). In TCC, loss of heterozygosity (LOH) on chromosome 9q and/or 9p is one of the most frequent genetic alterations (>50%) and is detected consistently in all stages and grades of tumours (Cairns *et al.*, 1993; Dalbagni *et al.*, 1993; Olumi *et al.*, 1990; Wu *et al.*, 1991) indicating that this may represent an early event in tumour development. This observation indicates that there are likely to be important tumour suppressor genes for TCC on 9p and/or 9q and one of these genes might represent a 'gatekeeper' for urothelial cells (Kinzler and Vogelstein, 1996). Detailed LOH studies of chromosome 9q in TCC have indicated that there are several candidate tumour suppressor loci (Cairns *et al.*, 1993; Dalbagni *et al.*, 1993; Olumi *et al.*, 1990; Simoneau *et al.*, 1999), one of which is at 9q32-33 (Habuchi *et al.*, 1995, 1997; Nishiyama *et al.*, 1999b). The putative tumour suppressor gene in this region has been designated *DBC1* (*d*eleted in *b*ladder *c*ancer *1*). Using YAC and PAC contigs spanning the *DBC1* region, we identified a candidate tumour suppressor gene, *DBCCR1* (*d*eleted in *b*ladder *c*ancer *c*hromosomal *r*egion candidate *1*) (Habuchi *et al.*, 1998; Nishiyama *et al.*, 1999a). *DBCCR1* encodes a putative protein of 761 amino acids with a predicted mass of 88.7 kDa. Mutation analyses of the coding region and Southern blot analyses detected neither somatic mutations nor gross genetic alterations in primary TCC. Although transcripts of *DBCCR1* are expressed in multiple normal human tissues including urothelium, its expression is suppressed by hypermethylation of the 5′ CpG island in 50% of bladder cancer cell lines investigated, suggesting that *DBCCR1* may be the target gene (Habuchi *et al.*, 1998). The function of *DBCCR1* remains elusive since it has no significant homology with known amino acid sequences and as yet no predicted structure. To address its candidacy as a tumour suppressor gene, we have used gene transfer methods to investigate the effects of *DBCCR1* protein expression on cell proliferation and apoptosis.

Results

Polymorphisms of DBCCR1

Previous studies identified three silent polymorphisms in the coding sequence of *DBCCR1* (Habuchi *et al.*, 1997); T 1036 C (Ser -> Ser), C 2044 A (Ile -> Ile), and T 2642 C (Leu -> Leu). Further sequencing of cDNA clones, human DNAs from the blood of normal volunteers and TCC tumour patients resulted in the identification of four further single base changes; T 1459 A (Arg -> Ser), G 1491 A (Arg -> His), A 1727 G (Thr -> Ala), T 1759 C (Cys -> Cys). Analysis of these changes in samples of normal volunteers and cDNA clones revealed that three of the changes were

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common polymorphisms; T 1459 A (33% of samples), A 1727 G (25%), and T 1759 C (60%). The fourth single base change (G 1491 A) was found only in cDNA clone ICRFp507K12270 (Habuchi *et al*., 1997) but not in 23 normal human genomic samples and six cDNA clones analysed. For functional analyses, we used a cDNA sequence containing none of these polymorphisms (wildtype) and a sequence containing G 1491 A and A 1727 G (polymorphic).

Molecular weight of DBCCR1 protein

Based on the predicted protein sequence of *DBCCR1*, the calculated mass was 88.7 kDa (Habuchi *et al*., 1997). To verify this, *DBCCR1* cDNA was fused with an HA epitope tag at the 5′-end, transfected into NIH3T3 cells and analysed by Western blotting using an anti-HA antibody. The relative mobility of HA-DBCCR1 was shown to be approximately 100 kDa (Figure 1a, left). Since this was larger than the predicted size, *DBCCR1* was fused with green fluorescent protein (GFP) at the 3′-end, transfected and examined by Western blotting with an anti-GFP antibody. This detected DBCCR1-GFP at 130 kDa and GFP alone at 30 kDa (Figure 1a, right), confirming the relative mobility of DBCCR1 to be 100 kDa.

Subcellular localization of DBCCR1 protein

The subcellular localization of DBCCR1 was investigated using transient transfectants of NIH3T3 expressing HA-DBCCR1. Immunofluorescence studies showed that DBCCR1 protein was localized primarily in the cytoplasm (Figure 1b,c). This observation was supported by analysis of NIH3T3 cells expressing a DBCCR1-GFP fusion in which the protein was also expressed only in the cytoplasm (Figure 1d,e).

Inhibition of NIH3T3 cell proliferation by DBCCR1

The effect of DBCCR1 on cell proliferation was investigated using NIH3T3 cells stably transfected with either epitope tagged HA-DBCCR1, non-tagged DBCCR1, antisense-DBCCR1, HA-DBCCR1-P (contains both G1491A and A1727G sequence variants), or vector alone (control). Cells were maintained in culture for 10 days following transfection before cell number and colony morphology were examined. Comparison of HA-DBCCR1 transfected cells with those transfected with vector alone (control) indicated that the majority of HA-DBCCR1 transfectant colonies were smaller in size than controls and showed altered morphology (Figure 2a,b). In addition, the number of viable cells in HA-DBCCR1 transfectant populations were about fourfold lower than those in control cultures (Figure 2c). Similarly, transfection of non-tagged DBCCR1 and DBCCR1-P resulted in growth suppression compared to controls (Figure 2c). Conversely, transfection with antisense-DBCCR1 did not result in growth suppression (Figure 2c). Large colonies that were obtained following HA-DBCCR1 transfection were morphologically similar to controls but when picked and expanded, did not show expression of the HA-DBCCR1 fusion protein by Western blotting. Smaller colonies were also picked but less than half of these could be expanded and only half of those which were expanded showed HA-DBCCR1 expression (data not shown).

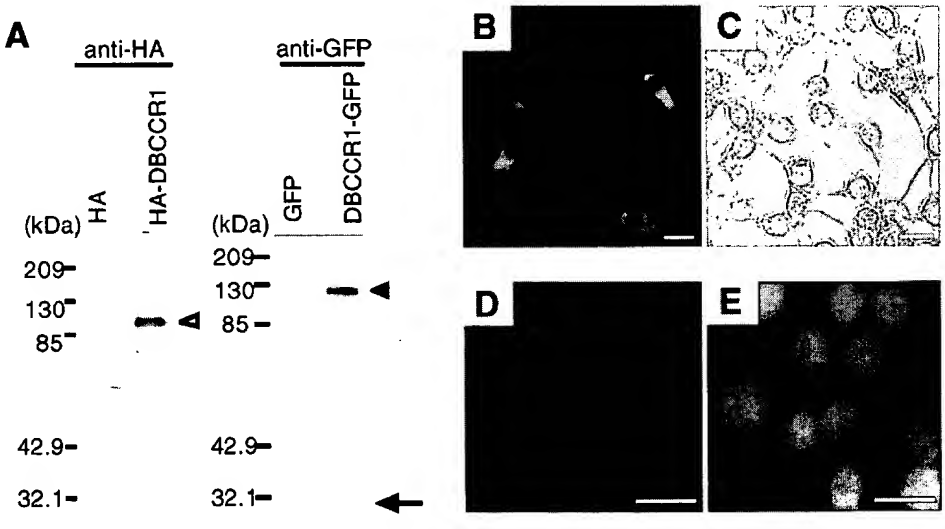

Figure 1 Expression of *DBCCR1*. (**a**) Western blot analysis of NIH3T3 cells transfected with HA-DBCCR1 or DBCCR1-GFP fusion protein constructs. Ten μg of protein lysates were resolved by SDS–PAGE, and detected with anti-HA antibody (left). Empty vector (pMKIT/HA) was transfected as a control (HA). DBCCR1-GFP was immunoprecipitated and analysed by Western blotting using anti-GFP (right). HA-DBCCR1 (white arrowhead); DBCCR1-GFP (black arrowhead); GFP (arrow). (**b–e**) Subcellular localization of exogenous DBCCR1. (**b**) Expressed HA-DBCCR1 was detected by immunofluorescence using anti-HA antibody followed by a FITC-labelled secondary antibody. (**c**) Phase contrast micrograph of the cells shown in (**b**). (**d**) DBCCR1-GFP transfectants. (**e**) Nuclei of cells shown in (**d**) stained using Hoechst 33258. White bar: 10 μm

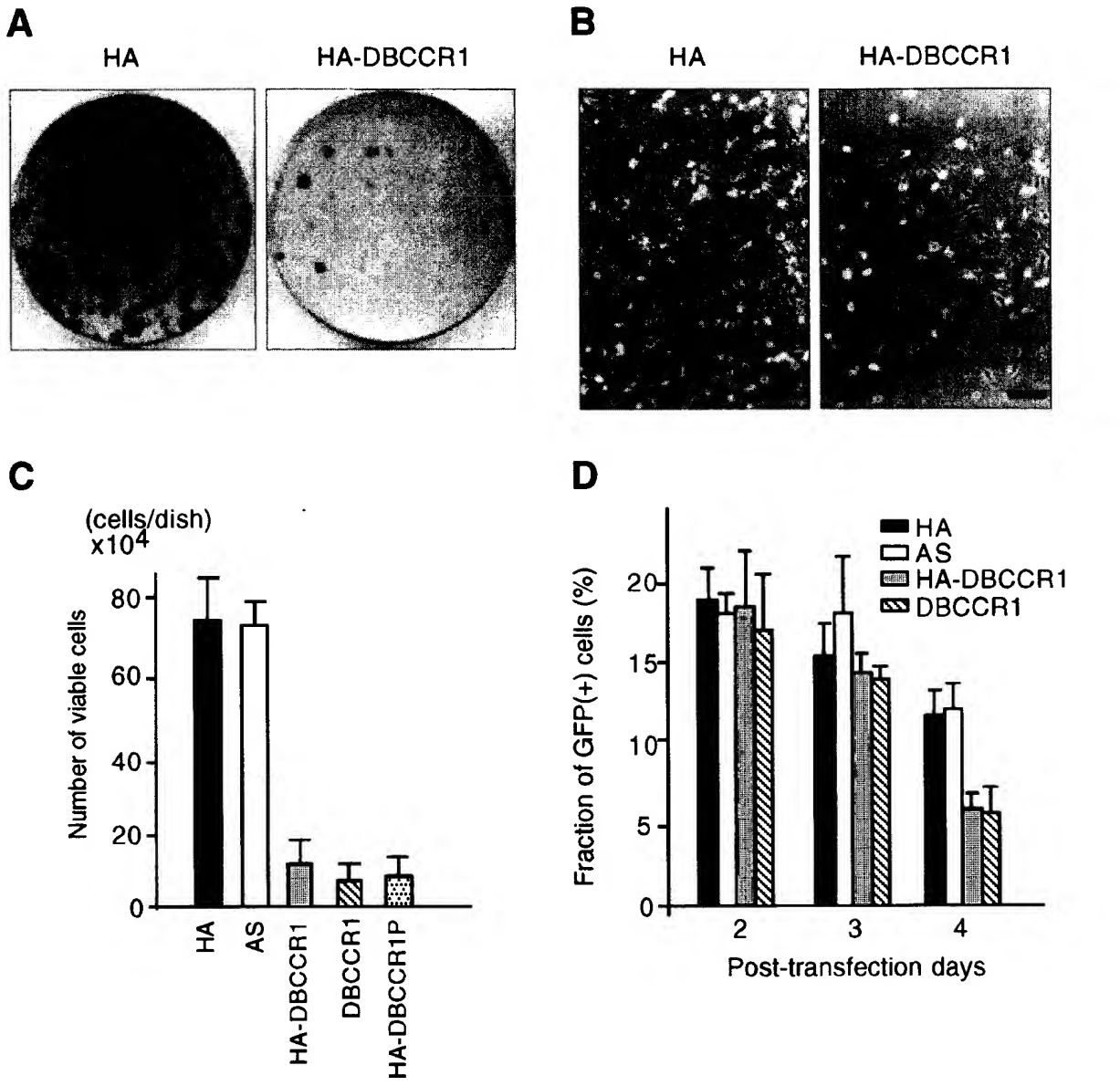

Figure 2 Suppression of NIH3T3 cell proliferation. (**a**) Methylene blue-stained colonies of HA-DBCCR1 transfectants after 9 days of G418-selection. Cells were transfected with either HA-DBCCR1 or control vector (HA). (**b**) Phase contrast micrograph of HA-DBCCR1 and control transfectants (HA) after 7 days of G418 selection. Bar: 50 μm. (**c**) Total cell numbers after 9 days of G418 selection. HA, control vector; DBCCR1, vector containing DBCCR1; HA-DBCCR1, HA-tagged DBCCR1; HA-DBCCR1P, HA-tagged polymorphic DBCCR1; AS, antisense orientated DBCCR1. (**d**) Reduction in the number of *DBCCR1* transfectants with time. Cells were harvested at the times indicated post-transfection and analysed by flow cytometry. The percentages of GFP-positive, co-transfected cells were measured. Each experiment was performed in triplicate, and a typical result depicted. Data was shown as mean $\pm$ s.d.

DBCCR1-induced growth suppression of NIH3T3 cells was also demonstrated using a transient co-transfection system. In this assay, an expression construct containing GFP targeted to the endoplasmic reticulum (ER-GFP) was co-transfected with either DBCCR1 or HA-DBCCR1 in order to monitor transfected cells by flow cytometry. The system was optimized using LacZ constructs in conjunction with ER-GFP. In this system $\geqslant$90% of ER-GFP positive cells also expressed LacZ (data not shown). Analysis of DBCCR1/HA-DBCCR1 transfectants demonstrated no difference in cell number compared to controls 24 h after transfection, but a dramatic decrease in the proportion of ER-GFP positive cells was observed at 4 days post-transfection compared to controls (Figure 2d).

Negative regulation of G_1/S transition by DBCCR1

To examine whether the reduction in proliferation was due to an effect on the cell cycle, the DNA content of

transfected cells was analysed using propidium iodide (PI) staining and flow cytometric analysis. Co-transfection of ER-GFP with the *DBCCR1* constructs was used to provide a fluorescent selection marker for successfully transfected cells and analysis was performed 72 h post-transfection. Transfection of vector alone did not affect cell cycle distribution. In contrast, transfection of HA-DBCCR1 resulted in an increase in the G_1 population of cells and a decrease in the number of cells in both S and G_2/M phases of the cell cycle (Figure 3a). A more precise assessment of the effects of DBCCR1 on the cell cycle was achieved by first synchronizing cells in a particular phase of the cycle. Serum-starvation of NIH3T3 cells for 24 h resulted in an accumulation of cells in G_1. Reintroduction of serum resulted in entry into S phase 16 h later (Figure 3b, GFP negative fraction). HA-DBCCR1 and control transfectants were analysed 16 h after release from serum-starvation-induced G_1 arrest. In both conditions, GFP negative cells showed almost the same cell cycle distribution. In contrast, HA-DBCCR1 transfectants were retained in G_1 (mean of three experiments; $83.8 \pm 5.53\%$), whereas control transfectants demonstrated a 'normal' cell cycle distribution (mean of three experiments; $46.2 \pm 7.79\%$; Figure 3b).

Cell cycle synchronization using the microtubule-stabilizing agent nocodazole causes cells to arrest in G_2/M phase. Treatment of NIH3T3 cells with nocodazole for 24 h resulted in $\geqslant 90\%$ of the cells accumulating in G_2/M. Following nocodazole treatment, cells transfected with either vector alone or antisense-DBCCR1 accumulated in G_2/M (mean of three experiments; $78.8 \pm 1.19\%$ and $76.0 \pm 2.92\%$, respectively). In contrast, cells transfected with either HA-DBCCR1 or DBCCR1 were retained in G_1 even after 24 h of nocodazole treatment (mean of three experiments; $58.1 \pm 3.35\%$ and $58.1 \pm 2.76\%$, respectively) (Figure 3c). These results reinforced the hypothesis that growth suppression by DBCCR1 is due to the retention of cells in G_1.

Effect of DBCCR1 on apoptosis

Since several tumour suppressor genes induce apoptosis in concert with an induction of cell cycle arrest, the effect of *DBCCR1* on the level of apoptosis was analysed. The level of apoptosis following *DBCCR1* transfection was measured using the TUNEL assay and compared to the level of apoptosis induced by two different agents, etoposide and nuclease treatment. Successfully transfected cells were identified by ER-GFP expression and analysed by flow cytometry. The incidence of apoptosis in both HA-DBCCR1 and DBCCR1 expressing cells was not significantly different to that observed in cells transfected with either antisense-DBCCR1 or vector alone (Figure 4). In contrast, both nuclease and etoposide treatments resulted in an induction of apoptosis (Figure 4). Therefore, *DBCCR1* does not appear to directly perturb apoptosis when exogenously expressed in NIH3T3 cells.

Effect of DBCCR1 *in human bladder tumour cells*

The bladder tumour cell lines EJ and 5637 have been shown previously to have lost one entire chromosome 9 homologue (Williamson *et al*., 1995). Neither shows expression of *DBCCR1* and this is associated with DNA methylation of the retained allele (Habuchi *et al*., 1998). The effect of *DBCCR1* expression was investigated following transfection with HA-DBCCR1 or control vector. These human cell lines show a lower transfection frequency than NIH3T3 which did not permit repetition of the flow cytometric analysis of transient transfectants described above for NIH3T3. Instead, stable transfectants were selected following transfection.

In EJ, fewer stable transfectant colonies were obtained following transfection with HA-DBCCR1 than with control vector (Figure 5a). Cell counts of mass populations of transfected cells after 6 days of selection in G418 showed a decrease in cell number of approximately 30% compared with controls but there was no significant morphological difference between DBCCR1 transfectants and controls. Attempts to pick and expand transfectant colonies were slightly less successful for DBCCR1 transfectants than controls (17/21 for DBCCR1 vs 19/21 for HA vector) but only four of the 17 DBCCR1 transfected clones showed expression of the HA-DBCCR1 fusion protein by Western blotting (data not shown). No significant difference in the rate of proliferation was found for stable DBCCR1-expressing clones (data not shown).

The effects of DBCCR1 in the cell line 5637 were more profound than in EJ. After 9 days of selection in G418, small colonies could be identified in DBCCR1 transfected cultures but these were much smaller than controls. The majority of these small colonies subsequently died and by 14 days after transfection only a very few colonies survived (Figure 5b). Expansion of these colonies has proved difficult and to date no further experiments have been possible on such clones.

Discussion

This study provides functional evidence that the candidate tumour suppressor gene *DBCCR1* identified within the *DBC1* region of deletion at 9q32-33 in bladder cancer has growth-suppressing activity. Although the precise cellular function of the gene remains to be elucidated, we have shown that its antiproliferative effect is mediated via modulation of the G_1 checkpoint.

Genomic alterations of chromosome 9, particularly deletions, are the most common genetic events in bladder cancer and several potential tumour suppressor loci have been localized on 9p and 9q. Molecular genetic and cytogenetic analyses indicate that a locus or loci on 9q are relevant at an early stage in the development of superficial papillary tumours. For example, in studies where multiple synchronous or metachronous papillary tumours from the same

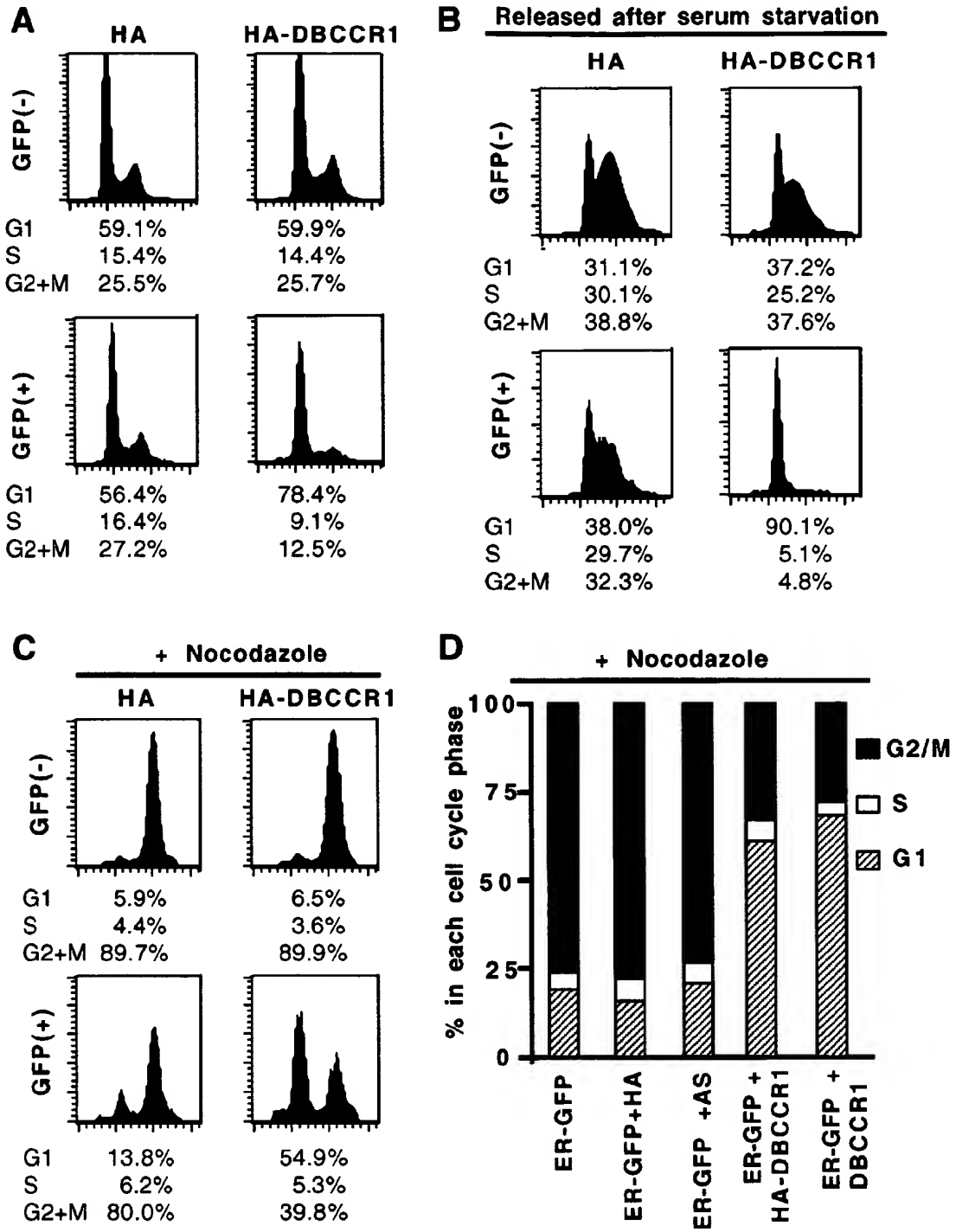

Figure 3 Flow cytometric analysis of *DBCCR1*-transfected NIH3T3 cells. (**a**) Cell cycle analysis of HA-DBCCR1 transfectants. Cells were co-transfected with control vector (HA) or HA-DBCCR1 expression vector and pBABE/GEM and harvested for cell cycle analysis 72 h post-transfection. (**b**) Cells were synchronized as described in Materials and methods and analysed by flow cytometry 16 h after release from cell cycle arrest. (**c, d**) Transfectants were synchronized in G_2 by nocodazole. Representative cell cycle profiles for control and HA-DBCCR1 transfectants are depicted in (**c**) and the cell cycle distributions in (**d**). Both GFP positive (transfected) and negative (non-transfected) cells were analysed. Experiments were performed in triplicate and a representative result shown

individual have been analysed, LOH of 9q has been the most frequent and consistent finding, as expected for an early event in tumour development. The presumed precursor lesion for papillary TCC is urothelial hyperplasia and a recent study has identified loss of chromosome 9 by FISH analysis in both histologically

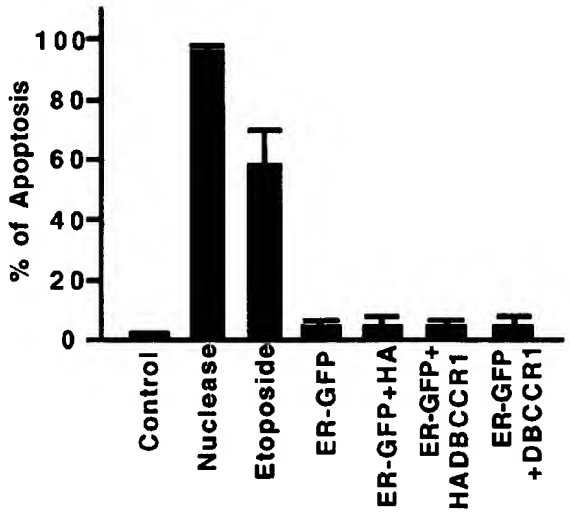

Figure 4 The effect of *DBCCR1* on apoptosis. Apoptosis was measured by TUNEL assay. The percentage of cells in apoptosis was determined by assessing the fraction of cells staining positive for nucleotide incorporation detected by flow cytometric analysis. Results are shown for untreated (control), nuclease or etoposide-treated (positive controls) and transfectants identified by GFP expression. Experiments were performed in triplicate. Data is shown as mean ± s.d.

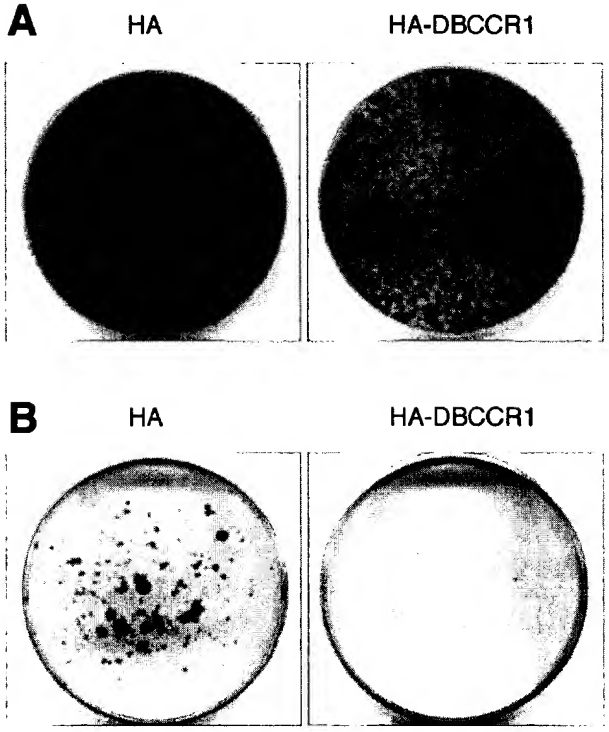

Figure 5 The effect of DBCCR1 in human bladder tumour cells. (**a**) Methylene blue-stained colonies of stable transfectants of EJ after 6 days of G418-selection. (**b**) Colonies of 5637 after 14 days of G418 selection. Cells were transfected with either HA-DBCCR1 or control vector (HA)

normal and hyperplastic biopsies from patients with a history of bladder cancer (Hartmann *et al.*, 1999). We postulate that the tumour suppressor(s) located in the *DBC1* region may have critical involvement early in bladder tumorigenesis since all the tumours identified with either small interstitial hemizygous or homozygous deletion in the *DBC1* region have been low grade superficial tumours (pTa, G1) (8, 9).

DBCCR1 is the only candidate gene so far identified within the relatively gene poor critical region of deletion at 9q32-33 (Habuchi *et al.*, 1997, 1998). This gene was shown previously to be silenced by methylation in 50% of bladder cancer cell lines (Habuchi *et al.*, 1998) and to be homozygously deleted in a primary tumour (Nishiyama *et al.*, 1999b) but small intragenic tumour-specific mutations have not been identified. The non-conformity of this gene to the Knudson two-hit mechanism for tumour suppressor gene inactivation by mutation (Knudson, 1971) may indicate either that *DBCCR1* is not the critical target gene or that it represents one of the rapidly growing class of suppressor genes that do not show inactivation by two mutational events. For example, the second allele may be inactivated predominantly by epigenetic means such as methylation, as previously observed (Habuchi *et al.*, 1998). Alternatively, haplo-insufficiency may provide an adequate selective advantage at the cellular level. For instance, if the levels of a tumour suppressor protein and its cellular target are finely balanced such that perturbation of the levels of either one results in an alteration in a growth control pathway, then haploid levels of a tumour suppressor may confer an altered phenotype as demonstrated for $p27^{KIP1}$ and *PTEN* (Di Cristofano *et al.*, 1998; Fero *et al.*, 1998).

In the absence of mutation of the gene except by homozygous deletion, authentication as a tumour suppressor relies largely on functional analysis. NIH3T3 cells were used for initial transfections and cell cycle analysis because of the ease with which they can be transfected. We have shown that exogenous expression of *DBCCR1* resulted in the suppression of proliferation of NIH3T3 cells and that this was due to the accumulation of cells in the G_1 phase of the cell cycle. Synchronization of cells either by serum starvation (G_1) or nocodazole treatment (G_2/M) provided further support for G_1-retention by the DBCCR1 protein. Interestingly, DBCCR1 did not cause a complete G_1 cell cycle arrest, since a small proportion of the population expressing the protein (~20%) were located in other phases of the cycle. This may be due to lower expression levels of DBCCR1 protein in these cells, the presence of a sub-population of cells which were 'resistant' to the effects of DBCCR1 or may indicate that DBCCR1 causes a slower G_1-transition rather than G_1-arrest. In addition to the short term effects of DBCCR1 on the cell cycle, the observed difficulty in expanding DBCCR1-transfected clones and the failure of many of those that were expanded to express the protein, revealed a long-term DBCCR1-associated growth inhibition compatible with G_1-retention.

Since NIH3T3 cells express mRNA from the mouse *DBCCR1* homologue (J Gill, 2001, unpublished results)

we wished to relate our observations more directly to human bladder tumorigenesis. We therefore introduced the gene into two bladder tumour cell lines which show loss of one allele of the gene and methylation induced silencing of the retained allele. The effects in these cells were striking and included a significant inhibition of proliferation. Future experiments with inducible expression constructs should allow a more detailed examination of the precise effects of *DBCCR1* expression on the phenotype of these cells. It will be important to examine the effects of lower levels of expression of the gene, for example under control of its own promoter and to study the effects of modulation of gene expression in normal urothelial cells.

Western blotting and immunofluorescence studies demonstrated that DBCCR1 is a 100 kDa protein localized in the cytoplasm. This sub-cellular localization of DBCCR1 suggests an indirect effect on the G_1 cell cycle machinery. Tumour suppressor genes have been demonstrated to regulate cell proliferation by perturbing both sides of the survival balance, resulting in a decrease in cell survival and an increase in cell death. For example, p53 induces both cell cycle arrest and apoptosis in a number of different cell types, the outcome being dependent on cell type and microenvironment (Sionov and Haupt, 1999). In contrast, $p16^{INK4A}$ functions to regulate G_1 cyclin-dependent kinase activity and thus proliferation (Roussel, 1999), but does not directly mediate cell death. Our data indicates that *DBCCR1* mediates G_1 cell cycle progression, but does not affect apoptosis either directly or as a result of prolonged G_1 retention (>24 h). This suggests that *DBCCR1* is more likely to impinge on a pathway upstream of a cell cycle 'gatekeeper' such as $p16^{INK4A}$ than upstream of a genomic 'gatekeeper' and 'caretaker' such as p53. One hypothesis is that it may act upstream of the G_1 checkpoint in a manner analogous to survival factors such as epidermal growth factor or negative factors such as transforming growth factor-beta$_1$ (TGFβ_1). For example, the TGFβ_1 pathway is known to cause a G_1 cell cycle arrest in many cell types by both upregulating G_1-cell cycle inhibitory molecules, $p15^{INK4B}$, $p21^{WAF1}$, $p27^{KIP1}$, and down-regulating levels of the cyclin dependent kinase 4 (CDK4) (Ewen *et al*., 1993; Ravitz and Wenner, 1997; Roussel, 1999). Thus, DBCCR1 may act as a 'checkpoint sentry', halting proliferation in order for other molecules to examine genomic fidelity, neoplastic potential and ultimately determine cell fate. Since DBCCR1 demonstrates no significant homology to any known amino acid sequence, (Habuchi *et al*., 1998), an exact role for this protein remains to be elucidated. Further analysis of these pathways and more efficient gene transfer methods such as retroviral or adenoviral-mediated gene transfer will be helpful in future functional studies.

Assessment of the frequency of alterations of expression of *DBCCR1* in primary tumours has proved difficult and it is not yet clear how many primary tumours with loss of one allele of *DBCCR1* by LOH have lost expression from the other allele via hypermethylation of the promoter. We have shown that 52% of bladder tumours have aberrant methylation of a region of exon 1 of *DBCCR1* containing 20 CpG dinucleotides (Habuchi *et al*., 2001). The frequency of methylation at certain specific CpGs however, is relatively low (Salem *et al*., 2000) and we have not found an absolute relationship of methylation with mRNA expression in cell lines (J Coulter and M Knowles, 2001, unpublished observations). Methylation levels therefore do not provide an adequate surrogate for expression that can be used in tumour DNA samples. A satisfactory antibody to the DBCCR1 protein has not yet been generated and levels of expression of the mRNA are too low to be detected by *in situ* hybridization. The possibility remains therefore, that loss of activity of one allele is sufficient for phenotypic alteration of urothelial cells. *In vitro* studies and the generation of *DBCCR1*-null mice may help to establish the possible effects of *DBCCR1* haploinsufficiency and confirm its role as a tumour suppressor.

Materials and methods

Cell culture and transfection

NIH3T3 cells were maintained in Dulbecco's modification of Eagle's medium with 2 mM L-glutamine and 10% foetal calf serum. The human bladder tumour cell lines EJ and 5637 were maintained in Dulbecco's modification of Eagle's medium and RPMI 1640 respectively, both supplemented with 2 mM L-glutamine and 10% foetal calf serum. Transfections were performed using Lipofectamine Plus Reagent (Life Technologies, Paisley, UK) following the manufacturer's protocol. Briefly, cells were seeded at 4×10^5 (NIH3T3), 3×10^5 (EJ) or 5×10^5 (5637) per 6 cm diameter dish 24 h prior to transfection with 2 μg of an expression construct. For co-transfection experiments, *DBCCR1* expression constructs and control plasmid (pBABE/GEM) were mixed in a 9 : 1 ratio and a total of 2 μg of plasmid DNA was used per dish. Stable transfectants were selected in G418 (Life Technologies) at 800 μg/ml (3T3) or 400 μg/ml (EJ and 5637).

Cloning of the DBCCR1 *gene and construction of plasmids for transfection*

pGreen Lantern-2 was purchased from Life Technologies. pCDNA3.1/*myc*-His and pCDNA3.1/*lacZ*/*myc*-His were purchased from Invitrogen (Groningen, Netherlands). pBABE/GEM, which expresses a green fluorescence protein targeted to the endoplasmic reticulum (ER-GFP) (Pestov *et al*., 1999), was a gift from Dr D Pestov (Department of Genetics, University of Illinois). pMKIT/HA was modified from pMKIT/neo to contain a haemagglutinin (HA) tag downstream of the SRα promotor (13). pMKIT/neo and pMKIT/HA were gifts from Dr K Maruyama (Tokyo Medical and Dental University School of Medicine) and Dr J Fujita (University of Kyoto).

The coding sequence of *DBCCR1* was obtained from EST ICRFp507k12270 (Higashitsuji *et al*., 2000) or p507k12270N, which was modified from ICRFp507K12270 to change A1491 to G and G1727 to A by PCR-based mutagenesis. To

generate *DBCCR1*-expression constructs, PCR amplification of the *DBCCR1* coding region was performed using primers DBC-F (5'-ACGCGTCGACATGAACTGGAGGTTTGTT) and DBC-R (5'-ATAAGAATGCGGCCGCTTAGCA-GAGTTTGGCTGT). PCR was for 25 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min using AmpliTaq Gold DNA polymerase (Perkin Elmer, Warrington, UK). PCR products generated from ICRFp507K12270 or p507K12270N were ligated into the *SalI*–*NotI* site of pMKIT/HA to generate *DBCCR1* tagged with HA at the N-terminus, termed pMKIT/HA-DBCCR1P or pMKIT/HA-DBCCR1, respectively. To generate a non-tagged *DBCCR1* expression construct (pMKIT/DBCCR1), the *NaeI*–*NotI* fragment of *DBCCR1* was ligated into the *EcoRV*–*NotI* site of pMKIT/neo. To generate an antisense construct, the *NaeI*–*EcoRV* fragment of *DBCCR1* was ligated into the *EcoRI*–*EcoRV* site of pMKIT/HA in the anti-sense orientation. To generate a *DBCCR1*-GFP fusion protein, *DBCCR1* (nucleotides 380–2701) was fused with GFP (nucleotides 10–747, from pGreen Lantern-2) and ligated into pCDNA3.1. All constructs were sequenced to eliminate PCR-generated artefacts.

Western blot analysis

Western blot analysis was performed as previously described (Nishiyama *et al.*, 1997). Briefly, cells were transfected as indicated above and harvested 48 h after transfection by lysis in RIPA buffer [150 mM NaCl, 50 mM TrisHCl pH 7.4, 5 mM EDTA, 1% Triton X-100, 1% Na-deoxycholate, 0.1% SDS, 1 mM phenylmethylsuphonyl fluoride (PMSF), 50 mM NaF, and both proteinase inhibitor (1×) and protein phosphatase inhibitor cocktails (1×) (Sigma, Dorset, UK)]. Protein concentrations were determined using the BioRad protein assay (BioRad, Hemel Hempstead, UK). Ten μg of protein was loaded per lane of 8% SDS polyacrylamide gels (SDS–PAGE). Protein was blotted onto nitrocellulose membranes (Hybond™ECL™, Amersham, Buckinghamshire, UK) using a semi-dry blotting system (BioRad). Equal protein loading on the membrane was verified using Ponceau S staining (Sigma). Non-specific binding of antibodies to the membrane was blocked using 5% dried milk powder (BioRad) in PBS containing 0.2% Tween 20. The membranes were probed using either anti-HA monoclonal antibody (12CA5; (Niman *et al.*, 1983)) or anti-GFP monoclonal antibody (3E1; a kind gift from Dr S Geley, ICRF, UK), localized using anti-mouse secondary antibody conjugated to horseradish peroxidase (Dako, UK) and detected using enhanced chemiluminescence reagents and Hyperfilm ECL (Amersham).

Immunoprecipitation

Cells were transfected with either pCDNA3.1/DBCCR1-GFP or pCDNA3.1/GFP as detailed above and maintained in culture for 48 h. Cells (1×10^6) were lysed in immunoprecipitation (IP) sample buffer (50 mM TrisHCl pH 7.4, containing 5 mM EDTA, 0.1% Triton X, 100 mM NaCl, 1 mM PMSF, 10 mM NaF, 1 mM DTT, 10% glycerol and both protease inhibitor (1×) and protein phosphatase inhibitor cocktails (1×)). Lysates were incubated with anti-GFP antibody (3E1), cross-linked with protein G-sepharose and the complex precipitated. The pellet was solubilized in RIPA buffer with 6× loading dye and analysed by Western blotting, as detailed above.

Immunocytochemistry

Immunocytochemistry was performed as previously described (Nishiyama *et al.*, 1997). Briefly, transfected cells were re-seeded on multiwell slides (Hendley, Essex, Essex, UK) at 24 h post-transfection. Following a further 24 h in culture, cells were fixed in methanol:acetone (1 : 1) and labelled with a rabbit anti-HA antibody (Y11; Santa-Cruz Biotech., CA, USA). Primary antibody was localized using goat anti-rabbit Ig $F(ab')_2$ conjugated to FITC (Southern Biotechnology Associates Inc, Birmingham, USA). Cells were counter-stained with Hoechst 33258 (Molecular Probes, Eugene, USA) and viewed by fluorescence microscopy.

Measurement of cell proliferation

Transfected cells were seeded at 1×10^5 cells / 6 cm diameter dish at 24 h post-transfection and cultured for 10 days in growth medium containing G418 (800 μg/ml) for selection of stably transfected clones. Cell counts were performed using a haemocytometer and colonies were stained *in situ* with methylene blue.

Cell cycle analysis

Transfected cells were re-seeded in 10 cm diameter dishes at 24 h post-transfection and harvested for cell cycle analysis at 72 h post-transfection. Cells were synchronized in the cell cycle by either serum starvation (G_1 arrest), or nocodazole treatment (G_2 arrest). For serum starvation experiments, cells were maintained in 10% serum for 24 h, serum starved for 24 h and allowed to recover in medium with 10% serum for 16 h prior to harvesting and analysis. For nocodazole-induced cell cycle arrest, cells were maintained in serum supplemented medium for 48 h post-transfection and then treated with nocodazole (40 ng/ml) for 16 h.

Synchronized cells were harvested, washed in PBS and fixed in 70% ethanol at 4°C. Cells were then incubated in PI (15 μM; Sigma) and RNase (4 μg/ml; Boehringer Mannheim, East Sussex, UK) for 30 min. Cell cycle distributions (PI, red fluorescence) were analysed on a FACScan® flow cytometer (Becton-Dickinson, Oxford, UK). Ten thousand events were acquired per sample and data were analysed using CellQuest software (Becton-Dickinson). For further analysis, the transfected cell population (GFP positive; green fluorescence) were 'gated' and their cell cycle distribution analysed.

Measurement of apoptosis

The effect of *DBCCR1* on apoptosis was assessed by TUNEL assay using the FlowTACS *in situ* apoptosis detection kit (R&D systems, Abingdon, UK) following the manufacturer's protocol. Briefly, cells were co-transfected with expression constructs and pBABE/GEM as detailed above and maintained in culture for 72 h. Subsequently, adherent cells were trypsinized and combined with those harvested from the supernatant. The resulting cell pellet was fixed in 4% paraformaldehyde and cells permeabilized by incubation in Cytopore for 30 min at room temperature. Permeabilized cells were labelled with biotin-conjugated dNTP using terminal deoxy-transferase, detected with phycoerythrin (PE)-conjugated avidin and analysed by flow cytometry (red fluorescence). Transfected cells (GFP positive) were 'gated' and analysed. Treatment of cells with either etoposide (100 μM; Sigma) for 48 h or incubation of fixed cells with TACS nuclease (R&D Systems) for 10 min at 37°C prior to analyses were used as positive apoptosis-inducing controls.

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Length = 761

Plus Strand HSPs:

Score = 2152 (762.6 bits), Expect = 6.4e-222, P = 6.4e-222
Identities = 408/777 (52%), Positives = 540/777 (69%), Frame = +3

```
SEQ 35:    57 MIWRSRAGAELFSLMALWEWIALS-LHCWVLAVAAVSDQHATSPFDWLLSDKGPFHRSQE 233
              M WR     EL    + +W   I++    H        A +DQH +   FDWL+SD+GPFH S+
DBCCR1:     1 MNWRF---VELLYFLFIWGRISVQPSH----QEPAGTDQHVSKEFDWLISDRGPFHHSRS 53

SEQ 35:   234 YTDFVDRSRQGFSTRYKIYREFGRWKVNNLAVERRNFLGSPLPLAPEFFRNIRLLGRRPT 413
              Y  FV+R RQGF+TRYKIYREF RWKV N A+ERR+ +   P+PL PEF R+IRLLGRRPT
DBCCR1:    54 YLSFVERHRQGFTTRYKIYREFARWKVRNTAIERRDLVRHPVPLMPEFQRSIRLLGRRPT 113

SEQ 35:   414 LQQITENLIKKYGTHFLLSATLGGEESLTIFVDKRKLSKRAEGSDSTTNSSSVTLETLHQ 593
               QQ  + +IKKYGTH L+SATLGGEE+LT+++DK +L ++   S + T S     +E LHQ
DBCCR1:   114 TQQFIDTIIKKYGTHLLISATLGGEEALTMYMDKSRLDRK---SGNATQS----VEALHQ 166

SEQ 35:   594 LAASYFIDRDSTLRRLHHIQIASTAIKVTETRTGPLGCSNYDNLDSVSSVLVQSPENKIQ 773
              LA+SYF+DRD T+RRLH IQI++ AIKVTETRTGPLGC++YDNLDSVSSVL+QS E+K+
DBCCR1:   167 LASSYFVDRDGTMRRLHEIQISTGAIKVTETRTGPLGCNSYDNLDSVSSVLLQSTESKLH 226

SEQ 35:   774 LQGLQVLLPDYLQERFVQAALSYIACNSEGEFICKENDCWCHCGPKFPECNCPSMDIQAM 953
              LQGLQ++ P YLQE+FVQ+ALSYI CN EGE++C+ + C C C  +FP+CNCP  DIQ M
DBCCR1:   227 LQGLQIIFPQYLQEKFVQSALSYIMCNGEGEYLCQNSQCRCQCAEEFPQCNCPITDIQIM 286

SEQ 35:   954 EENLLRITETWKAYNSDFEESDEFKLFMKRLPMNYFLNTSTIMHLWTMDSNFQRRYEQLE 1133
              E  L  + ++W     D E SDEFK FMKRLP N+FL   +I   W  D + Q RY+ L+
DBCCR1:   287 EYTLANMAKSWAEAYKDLENSDEFKSFMKRLPSNHFLTIGSIHQHWGNDWDLQNRYKLLQ 346

SEQ 35:  1134 NSMKQLFLKAQKIVHKLFSLSKRCHKQPLISLPRQRTSTYWLTRIQSFLYCNENGLLGSF 1313
              ++ +    K Q+   KLF LS RC   P   LPR+RT   WL R+QS LYCNENG  G+F
DBCCR1:   347 SATEAQRQKIQRTARKLFGLSVRCRHNPNHQLPRERTIQQWLARVQSLLYCNENGFWGTF 406

SEQ 35:  1314 SEETHSCTCPNDQVVCTAFLPCTVGDASACLTCAPDNRTRCGTCNTGYMLSQGLCKPEVA 1493
               E   SC C     +C   +PC +G  ++C  C+  N + CG+CN GY L +G C+P+
DBCCR1:   407 LESQRSCVCHGSTTLCQRPIPCVIGGNNSCTMCSLANISLCGSCNKGYKLYRGRCEPQNV 466

SEQ 35:  1494 ES--TDHYIGFETDL--QDLEMKYLLQKTDRRIEVHAIFISNDMRLNSWFDPSWRKRMLL 1661
              +S  ++ +I FETDL  QDLE+KYLLQK D R+ VH  FISN++RL+++FDP WRKRM L
DBCCR1:   467 DSERSEQFISFETDLDFQDLELKYLLQKMDSRLYVHTTFISNEIRLDTFFDPRWRKRMSL 526

SEQ 35:  1662 TLKSNKYKSSLVHMILGLSLQICLTKNSTLEPVLAVYVNPFGGSHSESWFMPVNENSFPD 1841
              TLKSNK +   +HM++G+S++IC  +NS+L+P+  VYVNPF GSHSE W MP  E  +P
DBCCR1:   527 TLKSNKNRMDFIHMVIGMSMRICQMRNSSLDPMFFVYVNPFSGSHSEGWNMPFGEFGYPR 586

SEQ 35:  1842 WERTKLDLPLQCYNWTLTLGNKWKTFFETVHIYLRSRIKSNGPNGNESIYYEPLEFIDPS 2021
              WE+ +L    QCYNWTL LGN+WKTFFETVHIYLRSR +      NE+    P++  DPS
DBCCR1:   587 WEKIRLQNS-QCYNWTLLLGNRWKTFFETVHIYLRSRTRLPTLLRNET-GQGPVDLSDPS 644

SEQ 35:  2022 RNLGYMKINNIQVFGYSMHFDPEAIRDLILQLDYPYTQGSQ----DSALLQLLEIRDRVN 2189
              +   Y+KI+++QVFGYS+ F+ + +R  + Q++  YTQG Q     S +L LL+IRDR+N
DBCCR1:   645 KRQFYIKISDVQVFGYSLRFNADLLRSAVQQVNQSYTQGGQFYSSSVMLLLLDIRDRIN 704

SEQ 35:  2190 KLSPP---GQRRLDLFSCLLRHRLKLSTSEVVRIQSALQAFNAKLPNTMDYDTTKLC 2351
              +L+PP   G+ +LDLFSC+L+HRLKL+ SE++R+  AL  +N ++    D  T KLC
DBCCR1:   705 RLAPPVAPGKPQLDLFSCMLKHRLKLTNSEIIRVNHALDLYNTEILKQSDQMTAKLC 761
```

ScanProsite

Search a sequence against PROSITE

Sequence:

```
MIWRSRAGAE LFSLMALWEW IALSLHCWVL AVAAVSDQHA TSPFDWLLSD KGPFHRSQEY
TDFVDRSRQG FSTRYKIYRE FGRWKVNNLA VERRNFLGSP LPLAPEFFRN IRLLGRRPTL
QQITENLIKK YGTHFLLSAT LGGEESLTIF VDKRKLSKRA EGSDSTTNSS SVTLETLHQL
AASYFIDRDS TLRRLHHIQI ASTAIKVTET RTGPLGCSNY DNLDSVSSVL VQSPENKIQL
QGLQVLLPDY LQERFVQAAL SYIACNSEGE FICKENDCWC HCGPKFPECN CPSMDIQAME
ENLLRITETW KAYNSDFEES DEFKLFMKRL PMNYFLNTST IMHLWTMDSN FQRRYEQLEN
SMKQLFLKAQ KIVHKLFSLS KRCHKQPLIS LPRQRTSTYW LTRIQSFLYC NENGLLGSFS
EETHSCTCPN DQVVCTAFLP CTVGDASACL TCAPDNRTRC GTCNTGYMLS QGLCKPEVAE
STDHYIGFET DLQDLEMKYL LQKTDRRIEV HAIFISNDMR LNSWFDPSWR KRMLLTLKSN
KYKSSLVHMI LGLSLQICLT KNSTLEPVLA VYVNPFGGSH SESWFMPVNE NSFPDWERTK
LDLPLQCYNW TLTLGNKWKT FFETVHIYLR SRIKSNGPNG NESIYYEPLE FIDPSRNLGY
MKINNIQVFG YSMHFDPEAI RDLILQLDYP YTQGSQDSAL LQLLEIRDRV NKLSPPGQRR
LDLFSCLLRH RLKLSTSEVV RIQSALQAFN AKLPNTMDYD TTKLCS
```

PROSITE Release 18.8, of 28-Sep-2003

>PDOC00001 PS00001 **ASN_GLYCOSYLATION** N-glycosylation site [pattern] [Warning: pattern with a high probability of occurrence].

```
168 - 171  NSSS
337 - 340  NTST
456 - 459  NRTR
562 - 565  NSTL
609 - 612  NWTL
641 - 644  NESI
```

>PDOC00003 PS00003 **SULFATION** Tyrosine sulfation site [rule] [Warning: rule with a high probability of occurrence].

```
478 - 492  vaestdhYigfetdl
638 - 652  pngnesiYyeplefi
639 - 653  ngnesiyYeplefid
```

>PDOC00004 PS00004 **CAMP_PHOSPHO_SITE** cAMP- and cGMP-dependent protein kinase phosphorylation site [pattern] [Warning: pattern with a high probability of occurrence].

```
116 - 119  RRpT
154 - 157  RKlS
```

>PDOC00005 PS00005 **PKC_PHOSPHO_SITE** Protein kinase C phosphorylation site [pattern] [Warning: pattern with a high probability of occurrence].

```
 49 - 51  SdK
 72 - 74  StR
157 - 159  SkR
```

```
191 - 193  TlR
309 - 311  TwK
361 - 363  SmK
380 - 382  SkR
504 - 506  TdR
528 - 530  SwR
536 - 538  TlK
539 - 541  SnK
761 - 763  TtK
```

>PDOC00006 PS00006 **CK2_PHOSPHO_SITE** Casein kinase II phosphorylation site [pattern] [Warning: pattern with a high probability of occurrence].

```
  42 - 45  SpfD
218 - 221  SnyD
267 - 270  SegE
315 - 318  SdfE
418 - 421  SfsE
442 - 445  TvgD
523 - 526  SwfD
563 - 566  StlE
579 - 582  ShsE
592 - 595  SfpD
599 - 602  TklD
620 - 623  TffE
735 - 738  StsE
```

>PDOC00008 PS00008 **MYRISTYL** N-myristoylation site [pattern] [Warning: pattern with a high probability of occurrence].

```
142 - 147  GGeeSL
162 - 167  GSdsTT
414 - 419  GLlgSF
461 - 466  GTcnTG
577 - 582  GGshSE
694 - 699  GSqdSA
```

>PDOC00009 PS00009 **AMIDATION** Amidation site [pattern] [Warning: pattern with a high probability of occurrence].

```
114 - 117  lGRR
```

>PDOC50099 PS50311 **CYS_RICH** Cysteine-rich region [profile].

```
           The following hit is below threshold (may be spurious)
273 - 291  CkendcwchcgpkfpecnC
```

Graphical summary of hits *(java applet)*

ScanProsite

Search a sequence against PROSITE

Sequence:

```
MNWRFVELLY FLFIWGRISV QPSHQEPAGT DQHVSKEFDW LISDRGPFHH SRSYLSFVER
HRQGFTTRYK IYREFARWKV RNTAIERRDL VRHPVPLMPE FQRSIRLLGR RPTTQQFIDT
IIKKYGTHLL ISATLGGEEA LTMYMDKSRL DRKSGNATQS VEALHQLASS YFVDRDGTMR
RLHEIQISTG AIKVTETRTG PLGCNSYDNL DSVSSVLLQS TESKLHLQGL QIIFPQYLQE
KFVQSALSYI MCNGEGEYLC QNSQCRCQCA EEFPQCNCPI TDIQIMEYTL ANMAKSWAEA
YKDLENSDEF KSFMKRLPSN HFLTIGSIHQ HWGNDWDLQN RYKLLQSATE AQRQKIQRTA
RKLFGLSVRC RHNPNHQLPR ERTIQQWLAR VQSLLYCNEN GFWGTFLESQ RSCVCHGSTT
LCQRPIPCVI GGNNSCTMCS LANISLCGSC NKGYKLYRGR CEPQNVDSER SEQFISFETD
LDFQDLELKY LLQKMDSRLY VHTTFISNEI RLDTFFDPRW RKRMSLTLKS NKNRMDFIHM
VIGMSMRICQ MRNSSLDPMF FVYVNPFSGS HSEGWNMPFG EFGYPRWEKI RLQNSQCYNW
TLLLGNRWKT FFETVHIYLR SRTRLPTLLR NETGQGPVDL SDPSKRQFYI KISDVQVFGY
SLRFNADLLR SAVQQVNQSY TQGGQFYSSS SVMLLLLDIR DRINRLAPPV APGKPQLDLF
SCMLKHRLKL TNSEIIRVNH ALDLYNTEIL KQSDQMTAKL C
```

PROSITE Release 18.8, of 28-Sep-2003

>PDOC00001 PS00001 **ASN_GLYCOSYLATION** N-glycosylation site [pattern] [Warning: pattern with a high probability of occurrence].

```
156 - 159  NATQ
433 - 436  NNSC
443 - 446  NISL
553 - 556  NSSL
599 - 602  NWTL
631 - 634  NETG
677 - 680  NQSY
```

>PDOC00003 PS00003 **SULFATION** Tyrosine sulfation site [rule] [Warning: rule with a high probability of occurrence].

```
294 - 308  akswaeaYkdlensd
```

>PDOC00004 PS00004 **CAMP_PHOSPHO_SITE** cAMP- and cGMP-dependent protein kinase phosphorylation site [pattern] [Warning: pattern with a high probability of occurrence].

```
110 - 113  RRpT
522 - 525  KRmS
```

>PDOC00005 PS00005 **PKC_PHOSPHO_SITE** Protein kinase C phosphorylation site [pattern] [Warning: pattern with a high probability of occurrence].

```
 43 - 45  SdR
 66 - 68  TtR
104 - 106  SiR
178 - 180  TmR
```

```
359 - 361  TaR
367 - 369  SvR
409 - 411  SqR
468 - 470  SeR
527 - 529  TlK
530 - 532  SnK
545 - 547  SmR
644 - 646  SkR
661 - 663  SlR
757 - 759  TaK
```

>PDOC00006 PS00006 **CK2_PHOSPHO_SITE** Casein kinase II phosphorylation site [pattern] [Warning: pattern with a high probability of occurrence].

```
 23 - 26  ShqE
 56 - 59  SfvE
 83 - 86  TaiE
148 - 151  SrlD
296 - 299  SwaE
347 - 350  SatE
405 - 408  TflE
479 - 482  TdlD
514 - 517  TffD
554 - 557  SslD
570 - 573  ShsE
610 - 613  TffE
731 - 734  TnsE
```

>PDOC00007 PS00007 **TYR_PHOSPHO_SITE** Tyrosine kinase phosphorylation site [pattern] [Warning: pattern with a high probability of occurrence].

```
  4 - 10  Rfv.Ell.Y
```

>PDOC00008 PS00008 **MYRISTYL** N-myristoylation site [pattern] [Warning: pattern with a high probability of occurrence].

```
136 - 141  GGeeAL
431 - 436  GGnnSC
432 - 437  GNnsCT
684 - 689  GQfySS
```

>PDOC00009 PS00009 **AMIDATION** Amidation site [pattern] [Warning: pattern with a high probability of occurrence].

```
108 - 111  lGRR
```

>PDOC50099 PS50311 **CYS_RICH** Cysteine-rich region [profile].

```
          The following hit is below threshold (may be spurious)
260 - 278  CqnsqcrcqcaeefpqcnC
```

Graphical summary of hits *(java applet)*

ATCC

10801 University Blvd • Manassas, VA 20110-2209 • Telephone: 703-365-2700 • FAX: 703-

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF
THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT ISSUED PURSUANT TO RULE 7.3
AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.2

To: (Name and Address of Depositor or Attorney)

Human Genome Sciences, Inc.
Attn: James H. Davis
9410 Key West Avenue
Rockville, MD 20850

Deposited on Behalf of: Human Genome Sciences, Inc.

Identification Reference by Depositor:	**Patent Deposit Designation**
DNA Plasmid PS-113	PTA-909

The deposits were accompanied by: __ a scientific description _ a proposed taxonomic description indicated above.
The deposits were received November 2, 1999 by this International Depository Authority and have been accepted.

AT YOUR REQUEST: X We will inform you of requests for the strains for 30 years.

The strains will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a U.S. Patent is issued citing the strains, and ATCC is instructed by the United States Patent & Trademark Office or the depositor to release said strains.

If the cultures should die or be destroyed during the effective term of the deposit, it shall be your responsibility to replace them with living cultures of the same.

The strains will be maintained for a period of at least 30 years from date of deposit, or five years after the most recent request for a sample, whichever is longer. The United States and many other countries are signatory to the Budapest Treaty.

The viability of the cultures cited above was tested November 10, 1999. On that date, the cultures were viable.

International Depository Authority: American Type Culture Collection, Manassas, VA 20110-2209 USA.

Signature of person having authority to represent ATCC:

Barbara M. Hailey

Barbara M. Hailey, Administrator, Patent Depository

Date: November 11, 1999